Novel Approach of Isolation of Coumarin and Checking It's Efficacy Against Polymicrobial Septicaemia Infection

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of Master's in Microbiology

MASTER OF SCIENCE
IN
MICROBIOLOGY

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Acknowledgements

First and foremost, I would like to express my deepest gratitude to Almighty God for granting me the strength, patience, and determination to successfully complete this dissertation.

I am sincerely thankful to my supervisor, Dr. Mayukh Bose, for their invaluable guidance, constant encouragement, and insightful feedback throughout the course of this research. Their unwavering support and academic expertise have played a crucial role in shaping this work.

I would also like to extend my appreciation to the faculty and staff of the Department of Microbiology, Sister Nivedita University, for providing a stimulating academic environment and the necessary resources that contributed to my academic development.

My heartfelt thanks go to my parents and family members, whose unwavering love, sacrifices, and moral support have been my constant source of motivation. Without their encouragement, this journey would not have been possible.

I am also grateful to my friends and peers for their camaraderie, support, and for making this academic journey a memorable one. Special thanks to those who directly or indirectly assisted me with data collection, analysis, or provided thoughtful discussions that enriched my work.

Lastly, I would like to acknowledge all the participants, institutions, and individuals who contributed to the successful completion of this study. Your cooperation and input were essential and greatly appreciated.

Thank you all.

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Abbreviations

Short Form	Full Form
MIC	Minimal Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
IC 50	Inhibitory Concentration to 50% population
SAR	Structure-Activity Relationship
TLC	Thin Layer Chromatography
OD	Optical Density
ZOI	Zone Of Inhibition
PAL	Phenylalanine Ammonia Lyase
UV	Ultra Violet
UV-Vis	Ultraviolet-Vision
TPP	Sodium Tripolyphosphate
NPs	Nano Particles
PDA	Potato Dextrose Agar

Abstract

Polymicrobial septicaemia remains a major health concern due to the increasing resistance of pathogens to conventional antibiotics. One such naturally occurring substance that has the potential to be a medication candidate is coumarin, which has low toxicity, stability, and solubility. This study presents a novel approach for the isolation and evaluation of coumarin, a bioactive compound extracted from Cinnamomum species, and assesses its antimicrobial efficacy against bacterial and fungal strains involved in polymicrobial infections. Numerous plants, as well as certain fungi and bacteria, contain coumarin, a basic scaffold that is widely distributed in nature. Cinnamon bark was procured from the local market, processed into fine powder, and subjected to ethanol-based extraction to isolate coumarin. The compound was purified and subsequently nano-coated to enhance its shelf life and stability. The antimicrobial potential of the nano-coated coumarin was assessed through Minimum Inhibitory Concentration (MIC) and IC_{50} assays against various pathogenic bacteria and fungi. Results demonstrated a significant inhibitory effect of coumarin on microbial growth, with promising dose-dependent responses. These findings suggest that coumarin, especially in its nano-formulated state, holds potential as an effective therapeutic agent for combating polymicrobial septicaemia. This study underscores the relevance of plant-derived compounds in the development of novel antimicrobial strategies.

Keywords: Coumarin, Chitosan nano polymer, Anti-microbial activity, Anti-fungal activity, MIC, IC50, Drug efficacy, Septicaemia.

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Introduction

Sepsis, sometimes referred to as blood poisoning, is a dangerous and potentially fatal systemic infection that happens when pathogenic microorganisms get into the bloodstream and spread throughout the body. One of the most challenging forms of septicaemia is polymicrobial septicaemia, which happens when multiple microbial pathogens, including fungi and both Gram-positive and Gram-negative bacteria, are present simultaneously. This illness is associated with increased antibiotic resistance, higher rates of morbidity and mortality, and complex clinical presentations that significantly complicate diagnosis and treatment. Multidrug-resistant pathogens are becoming more common, which has further complicated traditional treatment options and raised the need for novel antimicrobial medications with broad-spectrum efficacy and decreased resistance potential.

Because of their diverse variety of biological functions, coumarins are a potential class of natural chemicals that are garnering attention. Coumarin, pharmacologically significant chemicals come from natural sources in a wide variety of plants, such as tonka beans, sweet clover, cinnamon, and various citrus fruits. Coumarins are phenolic compounds generated from cinnamic acid. These are 2H-chromen-2-one, or coumarin, is a member of the benzopyrone class [43]. Coumarins were initially used as flavourings and fragrances and have long been valued for their pleasant aroma. However, in recent decades, a great deal of pharmacological research has shown that they have the potential to be used for a variety of therapeutic purposes, including anti-inflammatory, anticoagulant, antioxidant, anticancer, antiviral, and—most importantly—antimicrobial activities. In pharmaceutical industry coumarin used as precursor for various synthetic procedures. 4-hydroxycoumarins are found to be as Vitamin K antagonist type [30 , 54]. Coumarin is a therapeutic agent, found as a naturally occurring secondary metabolite in plants, bacteria, fungi, essential oils and can also be chemically synthesized. Coumarin is mostly composed of a benzene moiety fused with an alpha-pyrene ring known as benzopyrene [25]. They are low molecular weight, stable, soluble substances free of toxicity and unfavorable side effects. Coumarins have these and a number of other characteristics that make them a promising therapeutic option for a variety of bacterial and viral illnesses. Numerous natural, synthetic, conjugated, and hybrid candidate lead compounds using coumarin scaffolds have been investigated and are at various phases of the therapeutic development process [35] .

During the manufacture of coumarins, plants mostly use the phenylpropanoid pathway, which begins with the amino acid phenylalanine. After enzymatic conversion, cinnamic acid undergoes lactonization and hydroxylation to form the coumarin skeleton. Synthetic chemical and biotechnological techniques can be used to enhance or duplicate this process in order to produce coumarin derivatives with superior pharmacological properties. Developments in microbial engineering have also enabled the heterologous manufacture of coumarins using genetically modified microbes, providing a sustainable and scalable approach to the creation of bioactive chemicals with potential medicinal applications. Through the metabolism of phenylalanine, cinnamic acid, and shikimic acid, the primary route of coumarin biosynthesis

takes place [27]. A novel hybrid molecule with increased antioxidant activity can be produced by taking advantage of the coumarin nucleus's antioxidant potential. The creation of a novel class of chitosan derivatives with the coumarin nucleus is a recent illustration of this tactic [28]. In 1820, the aromatic organic bioactive chemical coumarin was originally discovered from the seeds of a higher blooming plant called Dipteryx odorata, also referred to as tonka beans. In this thesis, coumarin is extracted from cinnamon barks. Cinnamon is a highly valued spice that has been used for centuries in medicine, culinary, and culture. It is renowned for both its warm flavor and distinct scent. It is taken from the inner bark of Lauraceae trees, particularly those belonging to the species Cinnamomum. Cinnamon has been used in Egypt from 2000 BCE, according to records, and was prized for its usage in embalming and religious ceremonies. According to the Bible and other ancient texts, cinnamon was more valuable than gold. It played a major role in the early international trade networks between Asia, the Middle East, and Europe and was a vital commodity in the spice trade. During the Age of Exploration, European countries like the Portuguese, Dutch, and British competed for control of regions that produced cinnamon, particularly Sri Lanka, due to its economic importance. Cinnamon is used in a variety of savory and sweet cuisines, as well as in traditional medicine, beverages, and fragrances. It is a crucial part of many spice blends, such as garam masala, pumpkin spice, and Chinese five-spice powder. It has been used medicinally to treat stomach issues, colds, and inflammation. Its potential involvement in blood sugar management has been studied more recently. The primary bioactive ingredient in cinnamon, cinnamonaldehyde, is what gives the spice its distinct flavor and aroma. There is also eugenol, coumarin, and linalool. Some nations have restricted the use of coumarin in food since it can be detrimental to the liver and kidneys in excess. Coumarin is found in higher proportions in cassia cinnamon.Cinnamon continues to be an important spice all throughout the world. Because Ceylon cinnamon is considered to be of superior quality due to its sweeter flavor and reduced coumarin concentration, it is more expensive and in high demand among health-conscious consumers. In many cultures, cinnamon represents warmth, prosperity, and good health. In summary, cinnamon is more than simply a spice; it has a long history in cooking, medicinal, and trading. Its versatility and the depth it adds to the human experience across all cultures are demonstrated by its enduring appeal.

Globally, nanotechnology has gradually but significantly dominated a variety of industries. Particularly in the industrialized world, where nano-scale markets have quickly taken over in the last ten years, this rapid pace of technological innovation is evident. Since nanotechnology is now a general-purpose technology, it is not a novel idea. Active and passive nanoassemblies, general nanosystems, and small-scale molecular nanosystems are the four generations of nanomaterials that have surfaced and are employed in transdisciplinary scientific domains [71]. By bridging the gap between classical and quantum mechanics, nanotechnology creates a mesoscopic system. The production of natural nanoassemblies, including agricultural goods, nanomedicine, and medical industry treatment and diagnostic instruments, is carried out using this mesoscopic technology [37]. Drugs and diagnostic tools based on nanotechnology are currently reducing diseases that were previously incurable. Additionally, bulk industrial

production and manufacturing have been significantly impacted by this technology. Nanotechnology employs the reverse engineering principle, which occurs naturally, in place of producing materials by reducing vast quantities of material. It enables the production of goods at the nanoscale, like atoms, and subsequently creates goods that function at a larger scale [13]. As science and technology progress, the scientific community embraces products and technologies that are safer, cleaner, and more affordable than those of the past. Additionally, they are worried about the financial health of technology because the world's natural resources are depleting too quickly [48]. Therefore, nanotechnology offers a solution to this issue; it is transparent, cleaner, and less expensive than earlier mass bulking and heavy machinery, and it has the potential to be applied to all facets of life, primarily in the fields of nanomaterial sciences, nanoelectronics, and nanomedicine, which are ingrained in all facets of chemistry as well as the physical and biological realms [53]. Therefore, it is reasonable to assume that future generations will be required to study nanotechnology [16].

When discussing the vast domains of biological sciences, biotechnology, and medicine, it is impossible to overlook the origins of nanomedicine. In the larger context of nanobiotechnology, nanotechnology is already surpassing expectations. Successful uses of nanotechnology in medicine have consistently raised human life quality, leading to the emergence of a whole new field called nanomedicine. This has enabled researchers to develop improved methods for disease prevention, screening, treatment, diagnosis, and proactive healthcare measures [60]. With developments in tissue engineering, gene therapy, and nano-based genomics, these procedures may also include drug design, conjugation, manufacture, and effective delivery methods. Given this, it is reasonable to assume that nanomedicine will soon be the primary area of study for the upcoming generation of biologists, who will examine the potential benefits and hazards of this field [58].

Compared to the basic material from which they originate, nanosized materials may exhibit enhanced or surprising features. Numerous fabrication methods (top-down, crosslinking, microbial) and potential applications (electronics, textiles, and medicine) have been identified as a result of extensive study on inorganic and organic nanoparticles. However, it's important to note that their impact on the environment and human anatomy has been questioned. However, it is well established that polysaccharide-based NPs are safe for the environment and are not at all associated with issues of toxicity, biodegradability, or physiological stability. For instance, the natural polysaccharide chitosan is widely utilized in pharmaceutical compositions [32 , 70]. Derived from chitin, chitosan is the main component of fish scales, crustacean and insect exoskeletons, and fungal cell walls. It is a cationic polymer made of (1–4)-2-amino-2-deoxy-β-D-glucan that has garnered greater interest than its base polymer, chitin, because of its pH sensitivity, biocompatibility, and bioactive properties [42 , 47]. Their special qualities—small size, high surface area to volume ratio, stability at high temperatures, translocation into cells, and high responsiveness to living cells—make them useful for both diagnosis and treatment. Because of their capacity to react and aggregate with other nanoparticles in their environment, they come in a variety of sizes and forms.

Since then, scientists have conducted in-depth study on chitosan nanoparticles and created a variety of techniques that take into account a range of variables, including size, stability, drug loading capacity, and retention duration. Emulsification, precipitation, ionic or covalent crosslinking, or combinations of these methods are the fundamental methods utilized to create chitosan nanoparticles. The earliest technique for creating chitosan NPs that was documented in the literature used emulsification and crosslinking, which combined the aldehyde group of a crosslinking agent with the amino group of chitosan [69]. Another methodology based on covalent crosslinking is the reversed micelles (microemulsion) method, which is similar to emulsification and crosslinking. However, the generation of chitosan NPs is now aided by water-in-oil reverse micelle structures. The chitosan-containing micelle's core functions as a nanoreactor, forming chitosan NP by crosslinking. Precipitation-based techniques can also be used to create chitosan nanoparticles. Emulsification and precipitation are the foundations of the phase inversion precipitation technique. An aqueous solution of chitosan and an organic phase (dichloromethane and acetone) are combined with a stabilizer (poloxamer) to create the oil-in-water emulsion. To produce well-dispersed emulsion droplets that are nanoscale in size, high-pressure homogenization is used. Following the removal of methylene chloride from the emulsion by evaporation at room temperature and low pressure, acetone diffuses out of the droplets and NPs precipitate concurrently [33]. Ionic gelation is one of the most popular preparation techniques for chitosan nanoparticles, which have been studied for more than 20 years. Calvo et al. (1997) were the first to describe this technique [34]. Its foundation is ionic crosslinking, which occurs when inversely charged groups are present. For instance, the protonated amino groups of chitosan and negatively charged polyanion groups like sodium tripolyphosphate (TPP) [62]. After adding chitosan to an aqueous acidic solution (often an acetic acid solution), the TPP aqueous solution is added while being vigorously stirred. The mixture of positively-charged chitosan molecules is diffused with anionic molecules, and crosslinking takes place, resulting in the production of nanoparticles.

A significantly wider variety of Chitosan-based delivery vehicles has been made possible by the demonstrated capability of chitosan nanoparticles as drug delivery systems [36]. The loading of chitosan nanoparticles with drug molecules and DNA is made possible by the presence of the glycosidic link, amino, and hydroxyl functional groups. Sustainable chemistry can be used to synthesize chitosans without the use of hazardous organic solvents because they dissolve in acidic aqueous solutions [21]. Another benefit of employing chitosan nanoparticles is that they allow for the controlled release of medications in vivo through mucoadhesion [5]. Parenteral, ophthalmic, oral, pulmonary, nasal, buccal, and vaginal drug administration are only a few of the significant potential uses for chitosan nanoparticles. Other uses include tissue engineering, cancer treatment, and more [38].

These days, resistance patterns of harmful bacteria to commonly used medications and antibiotics are the norm, causing headaches in clinics and wreaking havoc on public health around the globe. Due to ongoing genetic advancements in pathogenic bacterial strains that result in resistance to drugs and antibiotics that were never used frequently, the problem is more serious than previously thought. Regrettably, newer resistant bacteria continue to evolve with

every new generation of antibiotics or newly introduced drugs. Bacterial consortia can acquire traits from both genetically similar and distant bacteria according to the inherent/intrinsic bacterial genetic exchange systems. An estimated 50,000 people of all ages are impacted by infectious diseases, which claim the lives of almost 17 million people annually [24 , 46]. As a result, the use of antibiotics quickly loses its potency, creating a need for and supply of desirable antibacterials. Thus, the creation of novel antibacterial medication candidates continues to be the top priority. A significant health issue is the rise in microbial infection cases due to the development of antibiotic resistance, which is caused by either microbial genome changes or an evolved mechanism of resistance of action.One of the most difficult problems facing the scientific community and all of humanity in the near future is most likely the fight against illnesses and bacteria that are resistant to drugs. Bacteria that are resistant to one or more antimicrobials from three or more antimicrobial classes are known as multidrug resistant (MDR) bacteria, while those that are resistant to none of the antimicrobials are known as very drug-resistant strains [4 , 6]. Fungal diseases are a well-known problem for plants and animals, but they pose a more covert threat to human health. Species from one of four genera account for over 90% of all documented fungal-related fatalities: Pneumocystis, Aspergillus, Candida, and Cryptococcus [7 , 19]. With its antifungal properties, coumarin derivatives have prospective use in the pharmaceutical and agri-food industries. A reasonable strategy for designing and developing novel drugs is to manipulate the molecules of prospective lead compounds that have the same biological activities. This entails combining two distinct pharmacophore groups with comparable activity to create a hybrid lead candidate. Using mainstream medicinal chemistry techniques, natural compounds, especially phytochemicals like vanillin, thymol, eugenol, menthol, umbelliferon, carvacrol, curcumin, and a few others, are employed as antibacterial, antifungal, and anticancer agents [8 , 9 , 41].

Finding a candidate or candidates with strong antibacterial activity is the goal of this review, which aims to provide a succinct overview and specific highlights of coumarin structural variants with individually related schematic techniques. Diverse teams of chemists, biologists, and drug developers would be able to use this to identify and differentiate interesting structures that might be evaluated for future advancement in the creation of innovative therapeutic or antibacterial agents. Finding a new or unusual molecule and its derivatives to treat a variety of harmful viral diseases is still pharmacology's obsession. Using medicinal chemistry concepts to alter a phytochemical would be a novel strategy for potential future antibacterial applications. Recent studies have shed light on the antibacterial activity of coumarins, particularly against drug-resistant pathogens. They might function by rupturing microbial membranes, preventing quorum sensing, and interfering with the development of biofilms, a critical virulence element in polymicrobial illnesses. Both against specific bacterial strains and in polymicrobial settings, coumarins have demonstrated efficacy in addressing the complex pathophysiology of polymicrobial septicaemia. Additionally, certain coumarin derivatives exhibit synergistic effects when mixed with traditional antibiotics, suggesting that they may be used as adjuvants or as stand-alone antibiotics.

Comprehensive studies on coumarin's effectiveness against polymicrobial septicaemia are still scarce, despite its potential. Our knowledge of how coumarins act in the dynamic and hostile environment of polymicrobial bloodstream infections is severely lacking because the majority of current research focuses on in vitro investigations or monomicrobial models. It is both vital and timely to investigate coumarins as possible therapeutic agents given the intricacy of these illnesses and the growing problem of antibiotic resistance.

With a focus on how they might be utilized to treat polymicrobial septicaemia, this thesis aims to examine the mechanisms involved in the manufacture of coumarins, both natural and synthetic, and evaluate their antibacterial properties. By evaluating previous research, finding knowledge gaps, and conducting targeted trials, this study seeks to determine whether coumarins can serve as effective agents in the fight against this potentially fatal infection. In doing so, it contributes to the broader goal of developing novel, sustainable, and successful treatments for complex illnesses at a time when antibiotic efficacy is waning.

Review Of Literature

Although coumarins are found naturally in many plants, they are especially abundant in woodruff, licorice, strawberries, tonka beans, cinnamon, cherries, sweet clove, apricots, bison grass, and lavender. Since it was originally separated from coumarone in 1882, coumarin has been used in perfumes because of its pleasant (sweet) scent. It was originally synthesized in 1868. [1 , 26]

The kingdom of plants contains a vast diversity of chemicals known as coumarins. Coumarin concentrations are high in certain essential oils, such as cassia leaf oil, lavender oil, and cinnamon bark oil. Coumarins are also abundant in chicory and green tea. Seasonal variations and environmental factors can also affect the occurrence of coumarin in plant parts. [12]

Phenylpropanoids are produced via the natural biosynthesis of coumarins. The enzyme phenylalanine ammonia lyase (PAL) transforms phenylalanine into cinnamic acid, which in turn produces 4'coumaroyl-S-coA. A range of phenylpropanoids were then produced. Coumarin is produced by hydroxylation in the ortho position, which is followed by side chain isomerization and cyclization. [61]

Based on their chemical diversity and complexity, natural coumarins are classified into various classes, including simple coumarins, isocoumarins, furanocoumarins, pyranocoumarins (both linear and angular), biscoumarins, and additional coumarins including phenylcoumarins. [72]

As polymeric platforms for the creation of novel pharmacological and therapeutic drug release systems with enhanced biodistribution, higher specificity and sensitivity, and less pharmacological toxicity, chitosan nanoparticles have attracted a lot of attention. The nasal, oral, ocular, and pulmonary routes of drug delivery have all been found to be suitable for chitosan nanoparticles. Chitosan's ability to increase absorption makes these applications possible. Furthermore, chitosan nanoparticles have demonstrated adjuvant effects in vaccinations and have been suggested as non-viral vectors in gene therapy. Improved absorption and bioavailability of pharmaceuticals encapsulated in chitosan nanoparticles allow for the delivery of gene medications, protein therapeutics, and other substances while providing good in vivo protection against enzyme breakdown [44 , 52].

When used as a carrier, nanochitosan can increase the bioavailability of DNA and drugs, improve controlled sustained release of biomaterials, decrease toxicity, and be prepared in mild conditions without the use of an organic solvent, which prevents the destruction of DNA and drugs and leaves no residual solvent after the preparation process [11].

During preparation, chitosan nanoparticles can be employed to change the value of each parameter and modify protein loading. Additionally, they are easy to store and transport, have excellent stability, high protein packing efficiency, and may be made into a lyophilized powder [11]. For a variety of uses, the nanoparticles were kept as lyophilized powder. The deployment

of tumor vaccines has been impeded by the selective permeability of the cell membrane, which prevents the majority of biological macromolecules, including proteins, from entering cells [10].

Chitosan nanoparticles injected intravenously are likely effective against tumors because of their small particle size. It has been demonstrated that particle size is a crucial factor in achieving the best possible in vitro efficacy of chitosan nanoparticles. The surface-to-volume ratio and specific surface area may rise with decreasing particle size, increasing the dissolution and, consequently, the bioavailability of compounds that are weakly soluble in water. The effectiveness of particle-based oral medication delivery systems may be enhanced by small particles. Particle size reduction has also been used to improve the oral bioavailability of medications. Nanoparticles can boost the effectiveness of intravenous injections and extend the blood half-life of medications [45 , 63].

The relative nonspecificity of the majority of tumor chemotherapy medications is their main drawback. Since the medications are injected intravenously for widespread system dispersion, they attack both the target tumor cells and healthy, normal cells, which might lead to harmful side effects. The medications should ideally be localized to the tumor site. These days, chitosan nanoparticles are being altered for targeted and sustained/controlled release. Drug dosage, possible adverse effects on healthy tissue, and treatment expenses can all be decreased by attaching medications to magnetic particles. Particularly significant factors influencing blood circulation duration and the particles' bioavailability within the body include the magnetic particles' size, charge, and surface chemistry [2 , 15 , 17 , 65].

Anti-influenza, anticoagulant, antioxidant, anti-tuberculosis, antiviral, antimicrobial, anti-HIV, antineoplastic, neuroprotective, antihypertensive, anti-Alzheimer, analgesic, and antidiabetic properties are among the several activities exhibited by coumarin derivatives. The greatest option for treating multiple sclerosis is to use coumarins. [39 , 40]

A SAR investigation was conducted in 2005 to assess the antibacterial efficacy of around 50 coumarin derivatives, both synthetic and natural. The lowest inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of active compounds with MIC values ranging from 62.5 to 2000 μg/mL were used to assess bacterial sensitivity to coumarins. With a MIC of 62.5 μg/mL against S. aureus and B. cereus, osthenole, a naturally occurring coumarin with anti-inflammatory properties, demonstrated the strongest effectiveness among the active ingredients. [14]

With additional promising outcomes, Widelsky and colleagues isolated a few comparable linear furanocoumarins from Peucedanum luxurians Tamamsch fruits in 2018. Biological and pharmacological investigations on plant extracts and a few isolated chemicals have validated the antibacterial properties of plants in the Peucedanum genus, which have been utilized for centuries. [67]

Using the ionic liquid [Et3NH][HSO4] as a catalyst, Tiwari et al. synthesized fifteen new coumarin derivatives in 2017 without the need of solvents [56]. Compounds 60 and 61 proved

to be the most effective fungicides within the series after testing for both antifungal and antibacterial properties.

In a phytochemical study conducted in 2018, Liu et al. extracted 10 novel coumarin derivatives (3 monomers and 7 dimers) from Murraya exotica, a dwarf tree endemic to tropical and subtropical regions of Asia that has long been used to treat inflammatory illnesses. [31]

The coumarin scaffold's characteristics and applications are incredibly diverse. Several scientific and industrial fields have made use of coumarin-based compounds as active pharmaceutical ingredients, pesticides, fragrances, and dyes for a variety of applications, including laser technology, organic photoredox catalysis, cell imaging, photocleavable protective groups, and fluorescent biological probes. [3 , 18 , 20 , 22 , 59 , 68 , 72]

Photolysis of coumarins, especially 4-hydroxymethyl derivatives, is known to occur. In light of this idea, a number of interesting biomolecules—mostly acyl derivatives—have been connected to the coumarin nucleus. The biomolecules can then be released in biological systems when exposed to UV light. The mechanism of fusion, the chemical makeup of extra rings, and the existence of substituents that donate and remove electrons are some of the elements that govern the photophysical properties of the derivatives that are created. [55]

The discovery of nanoparticles' special qualities in recent years has made it possible to use them in a wide range of applications, including biomedicine, drug and gene delivery, tumor detection, tumor vaccines, tissue engineering, MRI contrast enhancement, sensor development, fluorescent biological labels, pathogen detection, protein detection, cell and biological molecule separation and purification, DNA structure probing, environmental remediation, and water purification [29 , 48 , 49 , 50 , 51 , 57 , 65 , 66].

Materials and Methods

Sample Preparation

Materials Needed:

1. Dried cinnamon bark
2. Mortar and pestle
3. Weighing balance
4. Sieve

Procedure:

1. Ensure the cinnamon bark is fully dried.
2. The cinnamon bark is ground into a fine powder using mortar and pestle.
3. The powder is passed through a sieve for uniform particle size.
4. Weigh a suitable amount of powdered cinnamon for extraction (e.g., 5 gm)

Extraction

Materials Needed:

1. Ethanol (99%)
2. Beaker
3. Glass rod
4. Filter Paper
5. Conical flask

Procedure:

1. Powdered cinnamon (5 gm) is taken and mixed with 99% ethanol in a ratio of 1:9 (45 ml) and heavily stirred in a beaker for 15-20 minutes. Ethanol solubilize the coumarin along with other phenols and flavins in it.
2. The solution is filtered and the filtrate is kept.
3. The filtrate is taken in a petri dish and heated in a hot air oven at 60°C .
4. Upon evaporation black dust is seen which is scrapped on drying. The black dust is measured (0.426 g).

Figure 1

Isolation and Purification

Materials Needed:

1. Petri dish
2. Hot air oven
3. Distilled water
4. Weighing balance

Figure 2

Procedure:

1. The black dust is mixed with water in 1:9 ratio (0.426 × 9 = 3.834 ml) and again heated in the hot air oven.
2. Again on drying the resultant black dust is measured (0.376 g).
3. The resultant coumarin solvent forms from black dust by mixing it with 40% ethanol.

Biochemical Tests

Test for coumarin:

10% NaOH is taken in a test tube and few drops of sample are given in it.

Test for phenol:

5% FeCl3 is made on the base of NH4OH and few drops of the sample are given in it.

UV-Vis Spectroscopy

The OD of the coumarin solution is measured at 280 nm and 300 nm.

Thin Layer Chromatography

Materials Needed:

1. Silica
2. Coumarin sample
3. Chloroform
4. Ethanol
5. Small beaker
6. Slide
7. Pencil and ruler
8. Hot air oven
9. Distilled water

Figure 3

Procedure:

1. Powdered silica are mixed with distilled water to make a gel like concentration and a put on slides to make the TLC plates
2. This plates are then put into hot air oven so that they can dry up and become hard.
3. As the TLC plates are ready , a point is marked in the middle of the plate at 1cm from the bottom using pencil and ruler. The point is known as origin point.
4. A small amount of coumarin solution is applied onto the origin point using a capillary tube.
5. 20 ml solvent of 40% chloroform in ethanol is made in a beaker and the TLC plates are put in it in such a way that the origin point is dissolved in the solvent.
6. The opening of the beaker is closed and the solvent is allowed to rise until its 1 cm from the top.
7. The plate is then removed and the solvent front is marked.
8. The Rf value is then calculated.

Figure 4

pH Control

pH of the coumarin solution is maintained in between 7.9-8.1 so that most of the precipitate formed will be dissolved.

Nano Preparation

Materials Needed:

1. Chitosan
2. Distilled water
3. Acetic acid
4. Sodium
5. Triphenyl phosphate (TPP)
6. Toluene
7. Magnetic stirrer
8. Beaker

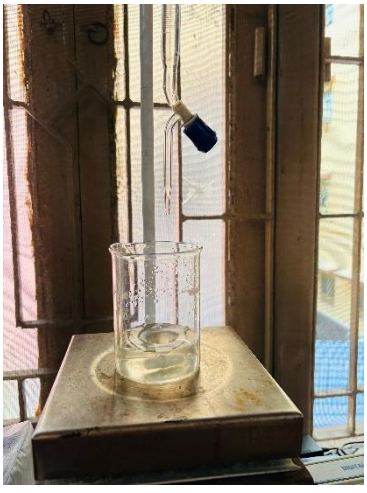


Figure 5

Procedure:

1. In a beaker 15 mg of chitosan and 15ml of distilled water are taken.
2. At room temperature, the solution is stirred on a magnetic stirrer for 1 hour.
3. In another beaker, 15 ml of distilled water is taken. 1 ml of distilled water is discarded and 1 ml of acetic acid is added.
4. Now this acetic acid solution is added dropwise to the chitosan solution and let it stirr for 30 minutes in magnetic stirrer.
5. 0.6 mg of TPP and 0.6 ml of toluene are mixed to form sodium tripolyphosphate.
6. This Sodium tripolyphosphate solution is added dropwise to the chitosan solution and again stirred for another 1 hour.

UV-Vis Spectroscopy

OD of the blank chitosan is measured. Peak is observed at a wavelength of 296 nm.

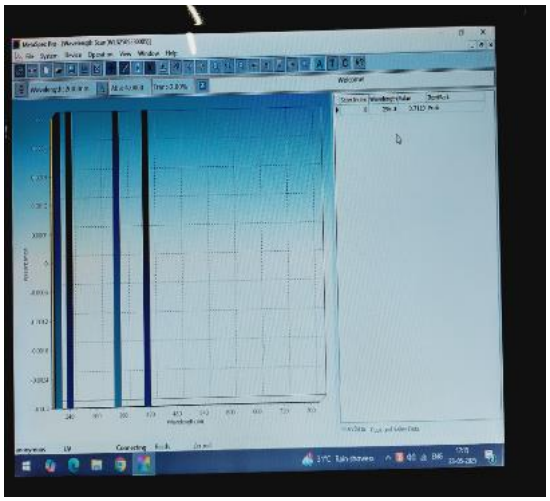


Figure 6

Coating of Drug with Nanopolymer

Figure 7

Materials Needed:

1. Pipette
2. Beaker
3. Measuring cylinder
4. Coumarin sample
5. Chitosan nanopolymer
6. Magnetic stirrer

Procedure:

1. As the volume of the coumarin sample is 11 mL , same volume of nanopolymer of 11 mL is measured and taken in a beaker.
2. The beaker is placed in a magnetic stirrer.
3. 11 mL of coumarin solution is added dropwise with the help of pipette.
4. The solution is stirred for 2 hours.

Stabilizing the Nano-Drug Conjugate

After coating, the pH of the sample is rised to 4.8 for maintaining its stability .

Figure 8

UV-Vis Spectroscopy

The OD of the drug is taken using a spectrophotometer. Peak is observed at a wavelength of 407 nm.

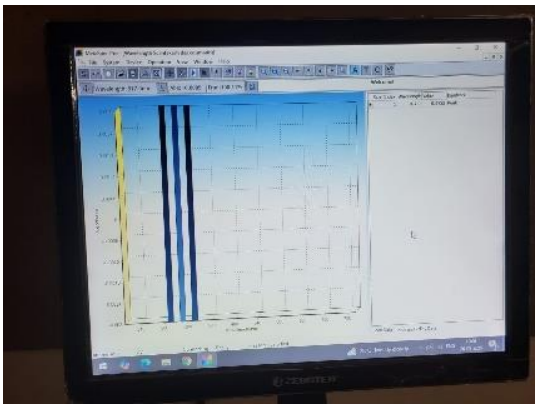


Figure 9

Preparation of Polymicrobial Sets

A polymicrobial set of bacteria refers to a mixture of multiple types of microorganisms, often including different species or strains of bacteria. Here, two polymicribial sets are prepared, set 1 and set 2.

In Set 1 microorganisms include: *Enterococcus faecalis, Listeria monocytogens, Serratia marcescens.*

In Set 2 microorganisms include: *Pseudomonas aeruginosa, Campylobacter jejuni, Listeria monocytogenes*.

Culture plate preparation

1. Preparation of LB media: For 10ml the composition of LB media include- Tryptone (0.1g), Yeast Extract (0.05g), Sodium Chloride (0.1g), Agar (0.15g).
2. After heating the agar media is ready to use.
3. Pour the agar media in Petri dish inside the laminar air flow, wait until it solidifies.
4. After solidification spread each bacterial strain in individual plate using a sterile glass rod or spreader as marked accordingly.

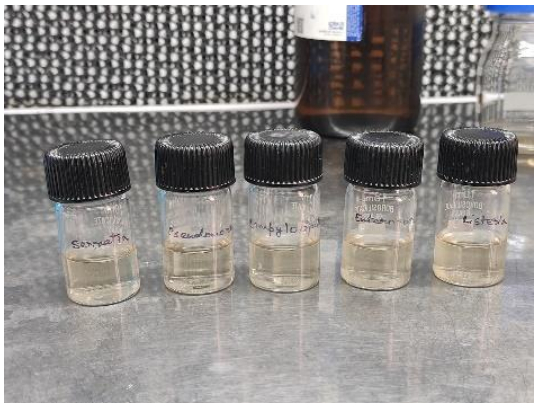


Figure 10

5. The plate is then incubated for overnight under appropriate conditions to allow for the growth of isolated colonies.

Polymicrobial set

1. LB broth is prepared as per quantity.
2. Inoculate each microorganism individually.
3. When all strains individual OD reached within a certain range of 0.6 then again, another LB broth is prepared.
4. The individual liquid cultures then add to the newly prepared broth according to the Set mentioned.

Figure 11

MIC of Bacterial Polysets

MIC of polyset 1 and polyset 2 are done in order to check if the drug sample can inhibit the growth of bacterial colonies. Dose calculation is done on the basis of concentration and various doses are given to find out its MIC.

Procedure:

1. When the standard's OD reached with in a certain range (0.56 - 0.62), MIC is performed.
2. 3 mL of standard solution is taken in a falcon.
3. A few volume of the standard solution is discarded, which is equal to the volume of the drug dose that is to be added using a pipette.
4. Then the drug dose is to added to the falcon and the the falcon is placed on a shaker.
5. OD readings are taken at an interval of 1 hour.

IC 50 of Bacterial polysets

IC_{50} of bacteria refers to the concentration of the drug required to inhibit 50% of bacterial growth or activity. IC 50 of polyset 1 and polyset 2 are done in order to find out the concentration of the drug sample where there is 50% reduction in the growth and activity of bacterial colonies. Dose calculation is done on the basis of concentration and various doses are given to find out its IC 50.

Procedure:

1. When the standard's OD reached with in a certain range (0.56 - 0.62), MIC is performed.
2. 3 mL of standard solution is taken in a falcon.
3. A few volume of the standard solution is discarded, which is equal to the volume of the drug dose that is to be added using a pipette.
4. Then the drug dose is to added to the falcon and the the falcon is placed on a shaker.
5. OD readings are taken at an interval of 1 hour.

ZOI of Bacterial Polysets

The Zone of Inhibition is the area around an antimicrobial agent where the growth of microorganisms is inhibited.

Procedure:

1. Prepare LB media and pour into Petri dish.
2. Allow the agar to solidify.
3. Spread the bacterial suspension using a spreader over the entire surface of the agar plate.
4. Use a sterile cork borer to punch well in the agar plate.
5. Remove the agar plug carefully to create a clean well.
6. Add 100µL of sample into the well.
7. Allow the plates to sit at room temperature for 30-60 minutes to let the agent diffuse into the agar.
8. Incubate the plates at 37°C for overnight.

PDA Media preparation for Fungus Culture

The composition of PDA media for 1000ml include – 200g Potato, 20g Dextrose, 20g Agar, Distilled water 1L**.**

Figure 12

Procedure:

1. Boiled diced potatoes in distilled water, then strain the mixture.
2. Add dextrose and agar to the potato extract.
3. Sterilize the mixture by autoclaving at 121 C for 15-20 minutes.
4. Pour the sterilized PDA medium into sterile petri plates.
5. Allow the medium to solidify.
6. Use a sterile spreader to spread the given fungus strain evenly spread across the plate.
7. Incubate the plate at suitable temperature for 3-5 days or until fungal growth is visible.

MIC of Fungus (*Aspergillus niger*)

The Minimum Inhibitory Concentration of a fungicide or antifungal agent is the lowest concentration that inhibits visible growth of a fungus.

Procedure:

1. Fungus culture is inoculated in the LB broth, and when the standard's OD reached within a certain range (0.56 – 0.62), MIC is performed.
2. 3 mL of standard solution is taken in a falcon.
3. A few volumes of the standard solution are discarded, which is equal to the volume of the drug dose that is to be added using a pipette.
4. Then the drug dose is to be added to the falcon and the falcon is placed on a shaker.
5. OD readings are taken at an interval of 1 hour.

IC 50 of Fungus (*Aspergillus niger*)

Procedure:

1. Fungus culture is inoculated in the LB broth, and when the standard's OD reached within a certain range (0.56 – 0.62), IC 50 is performed.
2. 3 mL of standard solution is taken in a falcon.
3. A few volumes of the standard solution are discarded, which is equal to the volume of the drug dose that is to be added using a pipette.
4. Then the drug dose is to be added to the falcon and the falcon is placed on a shaker.
5. OD readings are taken at an interval of 1 hour.

ZOI of Fungus (*Aspergillus niger*)

The Zone of Inhibition is the area around an antimicrobial agent where the growth of microorganisms is inhibited.

Procedure:

1. Prepare LB media and pour into Petri dish.
2. Allow the agar to solidify.
3. Spread the bacterial suspension using a spreader over the entire surface of the agar plate.
4. Use a sterile cork borer to punch well in the agar plate.
5. Remove the agar plug carefully to create a clean well.
6. Add 100µL of sample into the well.
7. Allow the plates to sit at room temperature for 30-60 minutes to let the agent diffuse into the agar.
8. Incubate the plates at 37°C for overnight.

Results and Discussions

Biochemical Tests

Test for Coumarin:

Experiment	Observation	Result
10% NaOH is taken in a test tube and few drops of sample are given in it.	Brown ring formation occurs.	Coumarin is present.

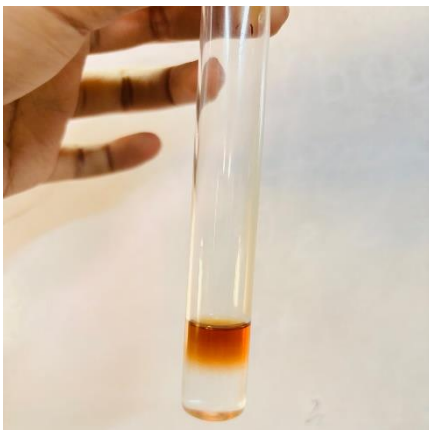


Figure 13

Test for Phenol:

Experiment	Observation	Result
5% FeCl3 is made on the base of NH4OH and few drops of the sample are given in it.	Black ring formation is seen.	Phenol is absent.

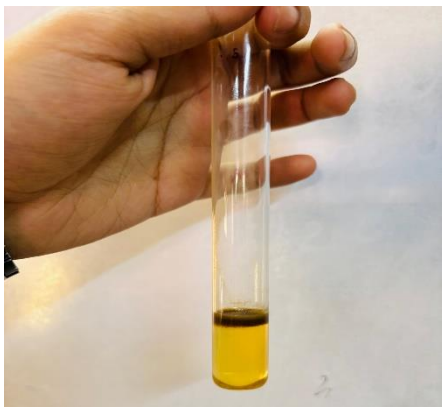


Figure 14

Thin Layer Chromatography

Distance covered by solvent = 6 cm

Distance covered by solute = 3.3cm

Therefore, Rf value = Ratio of the distance covered by the solute to that of the solvent

= 3.3 / 6 = 0.55

The Rf value for coumarin in chloroform solvent should be with in 0.5 - 0.7 range.

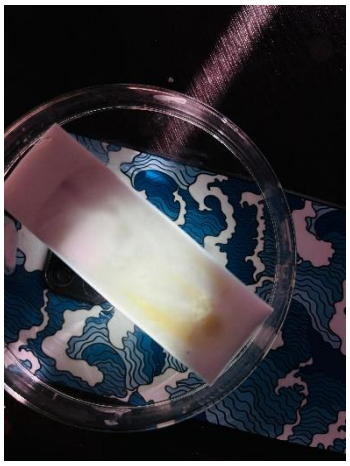


Figure 15

UV-Vis Spectroscopy

Wave length	OD
240 nm	0.860
300 nm	1.748

Calculation of sample concentration

Weight of sample = 0.426 g

Volume of sample = 11 ml

Therefore, concentration = (0.426 / 11) g/ml = 0.044181 g/mL = 44181 µg/mL

MIC of Bacterial Polysets

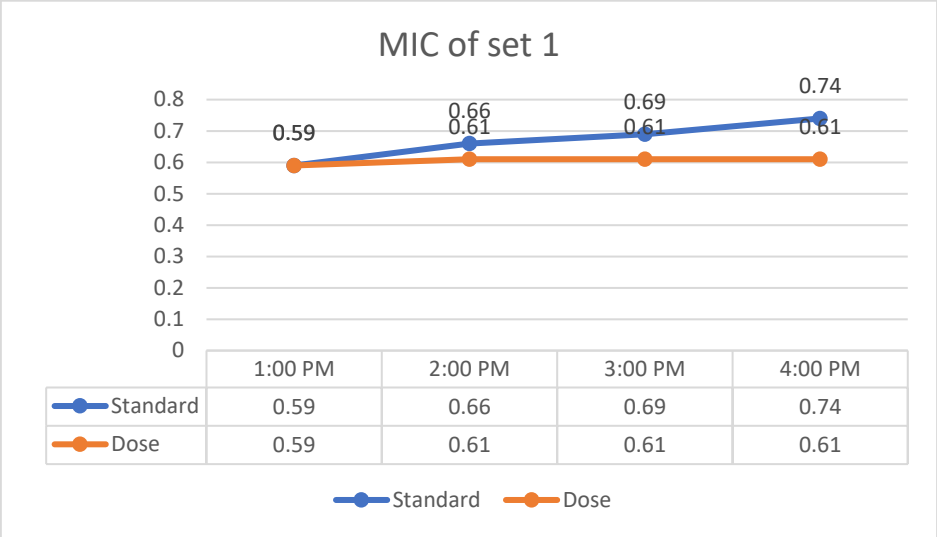
Set 1

C1 = 44181 µg/mL , C2 = 450 , V2 = 3 mL

V1 = 450 × 3 / 44181 = 0.02851 mL = 28.5 µL

Starting OD = 0.59 Dose = 28.5 µL Time interval = 1 hour

Standard OD	Dose OD
0.66	0.61
0.69	0.61
0.74	0.61



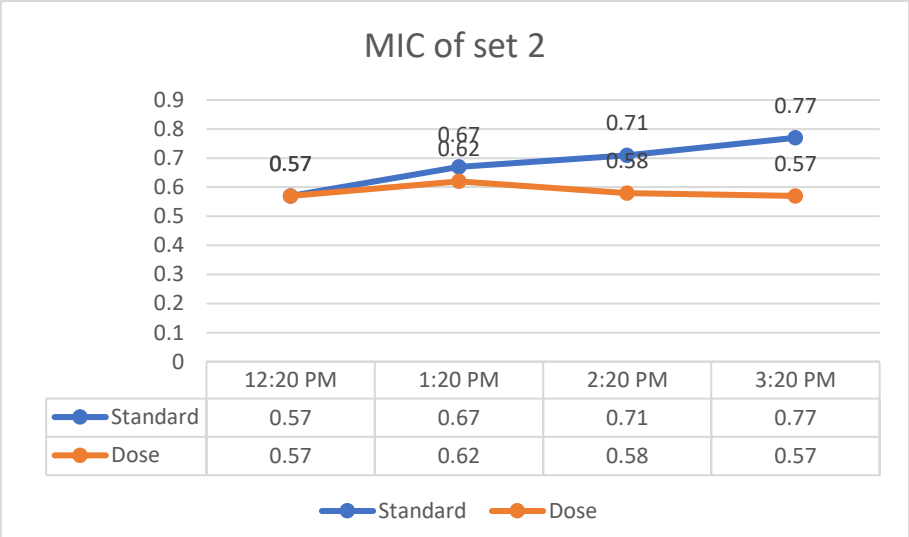
Set 2

C1 = 44181 µg/mL , C2 = 560 , V2 = 3 mL

V1 = 560 × 3 / 44181 = 0.03802 mL = 38 µL

Starting OD = 0.57 Dose = 38 µL Time interval = 1 hour

Standard OD	Dose OD
0.67	0.62
0.71	0.58
0.77	0.57



IC 50 of Bacterial Polysets

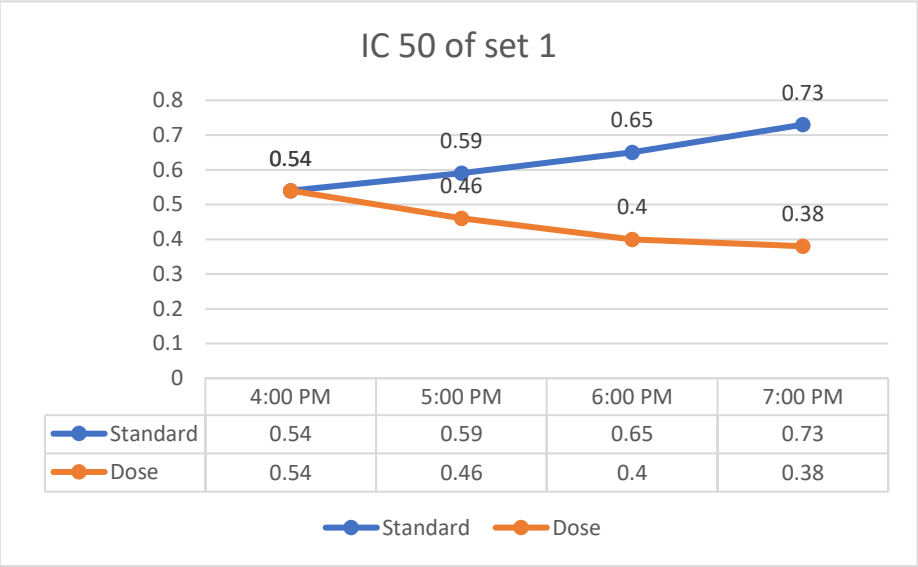
Set 1

C1 = 44181 µg/mL , C2 = 600 , V2 = 3 mL

V1 = 600 × 3 / 44181 = 0.04074 mL = 40.7 µL

Starting OD = 0.54 Dose = 40.7 µL Time interval = 1 hour

Standard OD	Dose OD
0.67	0.46
0.71	0.40
0.77	0.38



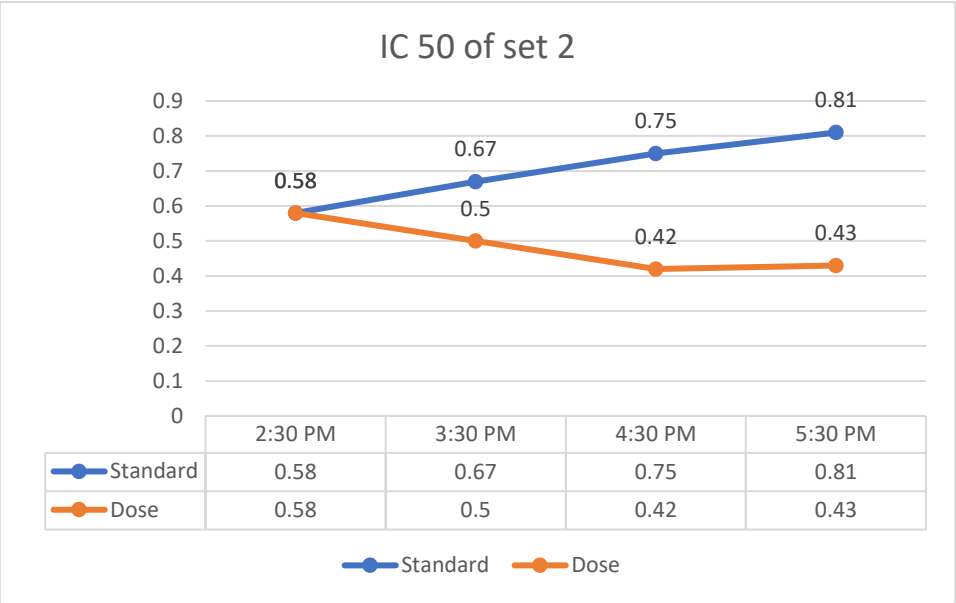
Set 2

C1 = 44181 µg/mL , C2 = 720 , V2 = 3 mL

V1 = 720 × 3 / 44181 = 0.04888 mL = 48.8 µL

Starting OD = 0.58 Dose = 48.8 µL Time interval = 1 hour

Standard OD	Dose OD
0.67	0.50
0.75	0.42
0.81	0.43



ZOI of Bacterial Polysets

The overnight culture inside the incubator forms a circular zone around the well where the drug was given. In this zone there is no bacterial growth seen. Now calculating the ZOI of both the sets;

Set 1

Diameter measured from one side = 1.8 cm

Diameter measured from another side = 1.8 cm

Diameter measured from other side = 2.0 cm

Average diameter = 1.87 cm

Diameter of the well = 0.8 cm

Therefore, ZOI = Average Diameter – Diameter of Well = (1.87 – 0.8) cm = 1.07 cm

Set 2

Diameter measured from one side = 2.2 cm

Diameter measured from another side = 2.1 cm

Diameter measured from other side = 2.1 cm

Average diameter = 2.13 cm

Diameter of the well = 0.8 cm

Therefore, ZOI = Average Diameter – Diameter of Well = (2.13 – 0.8) cm = 1.33 cm

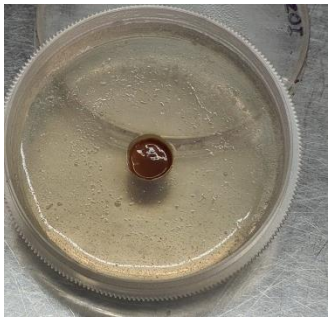


Figure 16

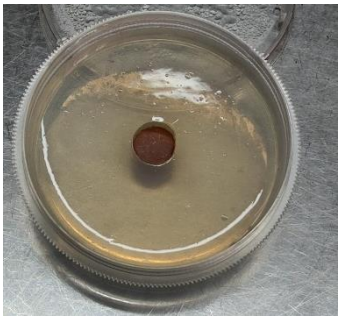


Figure 17

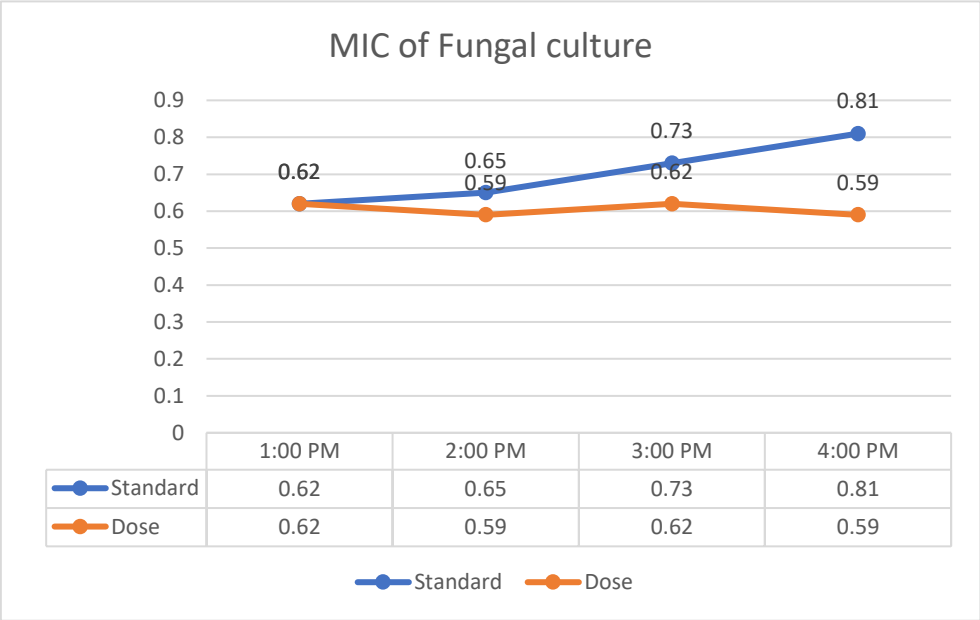
MIC of Fungal Culture

C1 = 44181 µg/mL , C2 = 210 , V2 = 3 mL

V1 = 210 × 3 / 44181 = 0.01426 mL = 14.2 µL

Starting OD = 0.62 Dose = 14.2 µL Time interval = 1 hour

Standard OD	Dose OD
0.65	0.59
0.73	0.62
0.81	0.59



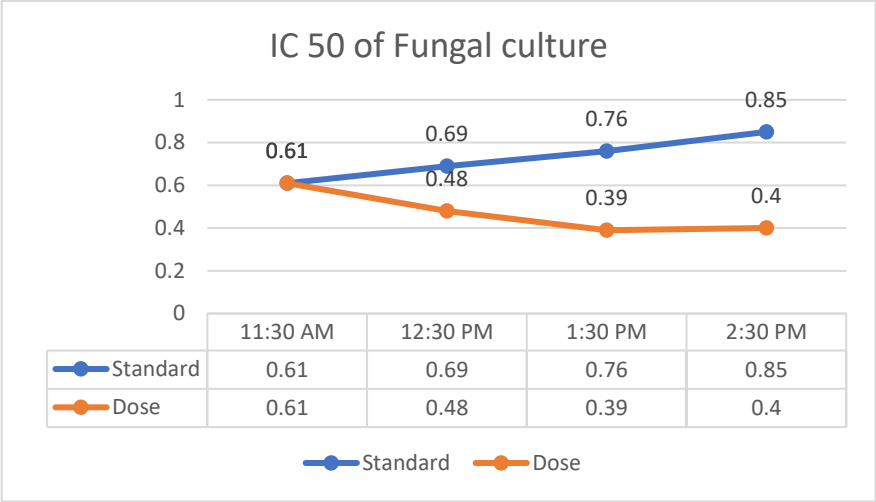
IC 50 of Fungal Culture

C1 = 44181 µg/mL , C2 = 400 , V2 = 3 mL

V1 = 400 × 3 / 44181 = 0.02716 mL = 27.1 µL

Starting OD = 0.61 Dose = 27.1 µL Time interval = 1 hour

Standard OD	Dose OD
0.69	0.48
0.76	0.39
0.85	0.40



ZOI of Fungal Culture

Diameter measured from one side = 1.8 cm

Diameter measured from another side = 1.8 cm

Diameter measured from other side = 2.0 cm

Average diameter = 1.87 cm

Diameter of the well = 0.8 cm

Therefore, ZOI = Average Diameter – Diameter of Well = (1.87 – 0.8) cm = 1.07 cm

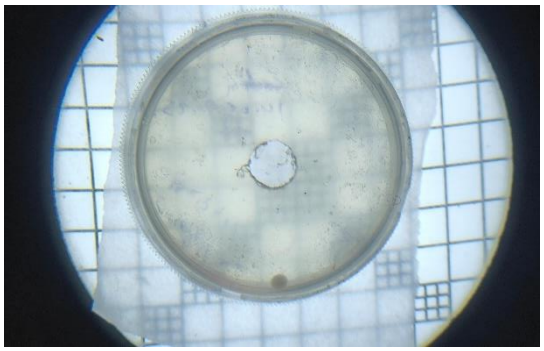


Figure 18

Conclusion

Given the increasing risk of polymicrobial septicaemia and the development in antibiotic resistance, it is imperative to develop novel, effective therapeutic strategies. This study successfully illustrated the potential of coumarin, a naturally occurring compound that was extracted from Cinnamomum species, as a promising antibacterial agent. An efficient extraction and purification process, followed by nano formulation utilizing chitosan-based polymers, significantly improved the stability and efficacy of coumarin. The nano-coated coumarin showed strong inhibitory effects against bacterial and fungal strains associated with polymicrobial illnesses, as indicated by the MIC and IC_{50} data.

The application of nanotechnology—more especially, chitosan nanoparticles—maintained a safe biocompatibility profile while also enhancing medication delivery and bioavailability. These findings demonstrate the importance of integrating phytochemistry and nanoscience to tackle the growing issue of multidrug resistance. Despite the promising results, additional in vivo and clinical research is required to validate the therapeutic potential of nano-coumarin formulations.

In conclusion, this study offers valuable new insights into the role of plant-derived compounds and nanotechnology in the development of modern antimicrobial drugs, laying the groundwork for future developments in the treatment of complex infectious diseases like polymicrobial septicaemia.

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